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Research Article

Production of lipid from depolymerised lignocellulose using the biocontrol yeast, *Rhodotorula minuta*: The fatty acid profile remains stable irrespective of environmental conditions

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Running Title: Microbial lipids from depolymerized lignocellulose

Keywords: biodiesel, lipid, yeast, Rhodotorula, Yarrowia lipolytica

Abbreviations: FAME, fatty acid methyl ester**, RSM** response surface methodology

Abstract

The oleaginous yeast *Rhodotorula minuta* has been used previously as a biocide agent and for the production of β-carotene. In addition, *R. minuta* has been shown to produce up to 40% lipids, while demonstrating a faster growth rate than the similar oleaginous yeasts; *Lipomyces starkeyii* and *Rhodotorula glutinis*. In this study this promising yeast was evaluated for its potential to produce glyceride lipids under the harsh conditions and complex sugar mixtures produced from depolymerised lignocellulose. The fatty acid profile of *R. minuta* was not found to change significantly irrespective of the environmental conditions and contained approximately 20% palmitic acid, 5% stearic acid, 60% oleic acid and 15% linolenic acid. *R. minuta* was found to grow on a range of sugars, and could consume xylose and glucose when both sugars were present, however, *R. minuta* was found to be highly sensitive to inhibitors, such as furfurals and organic acids, formed under the harsh lignocellulose depolymerisation conditions. Accordingly *R. minuta* did not grow well on biomass depolymerised with an acid pre-treatment stage. However, *R. minuta* was cultured successfully on food waste depolymerised with no additional acids, producing up to 19 g /L cell mass with a lipid content of up to 25% of the dry cell weight.

Practical applications

While high oil productivity, fast growth rates and a suitable fatty acid composition are key traits for the economic viability of SCO production, it is essential that the organism can be cultured and produce lipids from low-cost substrates. This requires the ability to assimilate the nutrients present in the desired hydrolysate, as well as maintain high growth rates in the presence of inhibitory compounds produced during the hydrolysis process. In this paper we demonstrate that the yeast *R. minuta* has industrial applicability in being able to convert depolymerized waste food to lipids with a profile akin to rapeseed oil.

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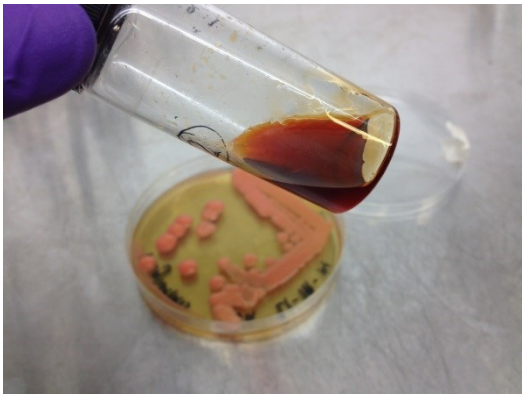
Graphical abstract



R. minuta lipid cultured on depolymerized food waste

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1 Introduction

Glyceride lipids are becoming an increasingly important feedstock for a number of industrial applications such as in the formulation of care products and in the production of biofuels. The vast majority of lipids are derived from edible plant oils such as rapeseed, sunflower and palm oil. However, increasing the production of these feedstocks, to provide for the growing demand, cannot be achieved without seriously impacting on food production. Due to this and the rising cost of production, a large research effort is currently underway to produce alternative glyceride feedstocks. One option is to produce these lipids from heterotrophic microbes, such as oleaginous yeasts, grown on depolymerised lignocellulose. Yeasts are attractive as they can reach high cell densities, have short doubling times and can produce a range of alternative side products [1, 2].

For non-oleaginous yeast, nitrogen limitation hinders cell growth and the excess carbon is channeled into polysaccharide synthesis [3]. In oleaginous yeast, nitrogen exhaustion initiates a series of metabolic steps leading to *de novo* lipid biosynthesis in which the carbon source is used for lipid accumulation rather than cell proliferation processes [4]. Lipid accumulation is influenced by the C/N ratio, with lipid accumulation induced at molar ratio C/N > 20 [5], but with an optimum being close to 100 [6]. For example the lipid yield from *R. glutinis* more than doubled when the C/N ratio was increased from 20 to 70, though a further increase did not lead to higher lipid yields [7]. While lipid contents of 60-70% of the cell are possible Ratledge *et al.* reasoned that due to the increased level of nutrients and decreased production of cell mass, the optimal level of lipid production is nearer 40% dry weight [8].

The environmental conditions also influence the fatty acid profile, which is important in determining the end-use of the lipids. Temperature-induced variations in the fatty acid profile of the yeast *C. oleophila, C. utilis* and *R. toruloides* have been demonstrated. Reducing the culture temperature for *C. curvatus* for example increased the amount of saturated esters by 10% [9] while the amount of polyunsaturates in various yeast of the Zygomycete genera were reduced substantially at lower growth temperatures [10]. In contrast, lower incubation temperatures were reported to increases the level of polyunsaturates in *R. glutinis* [11], and *Y. lipolytica* [12].

Depolymerised lignocellulose mainly contains the sugars glucose and xylose, though a large range of alternative sugars are present in lower concentrations. To increase the efficiency of the overall process it is vital that the oleaginous organism can metabolise the sugars available. Under the depolymerisation conditions, the sugars as well as residual lignin, can also break down to produce microbial inhibitors such as furfural, 5-hydroxymethylfurfural (5-HMF) and organic acids. These inhibitors are well known for their toxicity to microorganisms such as *S. cerevisiae* [13], though

tend to be less inhibitory to oleaginous yeasts [14]. While over 40 species of oleaginous organism are known the majority of research has focused on just a few yeasts such as *Rhodosporidium toruloides* [15,16]*, Yarrowia lipolytica* or *Rhodotorula glutinis* [17]*. R. glutinis* for example is known to produce up to 40% of the cell weight in lipid, has a high tolerance to inhibitors and can metabolise a large range of sugars [11].

A related species *R. minuta*, typically used for the production of carotenoids is also capable of producing glyceride lipids [18]. When optimally cultured on glucose, *R. minuta* was found to have a faster growth rate than either *R. glutinis* or *Lipomyces starkeyii* and was found to produce up to 48% of its dry weight in lipid [19]. *R. minuta* is commonly found growing on the skin of grapes and has been demonstrated to be an active biocide producing yeast [20]. We reasoned that this ability to ward off invasive biota coupled to the harsh acidic conditions where the yeasts thrive would make *R. minuta* a more suitable organism for a biotechnological application. In this study *R. minuta* was investigated for its ability to produce suitable lipids for fuel production from multiple sugar sources and under the harsh conditions created from depolymerised lignocellulose. Finally the efficacy of the yeast was investigated by culturing on depolymerised miscanthus, wheat straw and food waste.

2 Materials and methods

All chemicals and solvents were purchased from the Sigma-Aldrich chemical company. All reactants were used as received with no additional purification.

2.1 Microbial cultivation

Rhodotorula glutinis 2439, *Y. lipolytica* and *R. minuta* 62 were all purchased from the National Collection of Yeast Cultures (NCYC, Norwich, UK). The yeast strains were maintained on yeast peptone dextrose (YPD) agar plates [10 g/L yeast extract, 20 g/L peptone, 20g/L glucose, 15 g/L agar] at 4 °C until used. For the seed culture, 25 ml of YM medium [3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, pH 6.5] was inoculated with a single colony and were incubated at 28 °C and 180 rpm for 25 hours.

2.2 Cultivation for Response Surface Methodology (RSM) studies

Shaking flask cultures were carried out in 250 ml Erlenmeyer flasks containing 100 ml medium. The cultures were initiated with 10% (v/v) seeding culture into the original *R. minuta* (RM) medium [1 g/L yeast extract, 0.1 g/L NaCl, 2 g/L $(NH_4)_2SO_4$, 0.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl_2$], pH 6.3, and incubated in a rotary shaker at 180 rpm. The resulting cell mass was harvested after 120 h of growth. RSM using a 3^3 full factorial design was performed to develop mathematical correlations between three independent variables and to approach the optimum response region. The range of the variables tested was: glucose, 10-30 g/L; $(NH_4)_2SO_4$, 0.5 - 1.5 g/L and temperature, 25 - 35 °C. According to this design, 30 runs were conducted including three replicates at the central point for assessing experimental variance. Validation of the cultures was performed at 28 °C with 1) 15 g/L glucose, 0.75 g/L $(NH_4)_2SO_4$; 2) 25 g/L glucose, 0.75 g/L $(NH_4)_2SO_4$; 3) 15 g/L glucose, 1.25 g/L $(NH_4)_2SO_4$; 4) 25 g/L glucose, 1.25 g/L $(NH_4)_2SO_4$. The relationship of the variables was determined using the MATLAB model-based calibration, design of experiments software to fit a radial basis function – multiquadratic (qRBF) regression analysis to the experimental data. MATLAB SIMULINK model was then used to construct a process flow model to predict the outcome of lipid production for different growth conditions.

2.3 Diauxic growth cultures

The 26-hour-old seed cultures were centrifuged to pellet the cells (6000 rpm, 10 min) and the supernatant discarded to prevent the transfer of residual sugar. The cell pellet was resuspended in 25 ml RSM medium containing 1 g/L $(NH_4)_2SO_4$ but without sugar. 5 ml of this cell suspension was inoculate into 45 ml RSM medium containing 1 g/L $(NH_4)_2SO_4$ and 30 g/L of sugar (glucose and xylose) at the given weight ratios, in 100 ml Erlenmeyer flasks. The cultures were incubated at 28°C, 180 rpm for 120 h. All cultures were repeated in triplicate.

1 ml samples were removed every 24 h and the $O.D._{600nm}$ measured using a spectrophotometer (Spectronic 200, Thermo Scientific UK). The yeast cell mass was removed by centrifugation (14000

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rpm, 2 min) and the sugar uptake was assessed by reverse phase HPLC using a Shimadzu 10AVP HPLC system (Shimadzu corp., Japan) equipped with a RID-10A refractive index detector. All samples were filtered through a 0.20 µm filter membrane (Millipore, UK) before analysis. 20 µl of the sample (diluted accordingly) was injected onto an Aminex HPX-87H column (BioRad, CA, USA) and eluted isochratically over a 15 min period with 0.6 ml/min of 0.2 µm-filtered and degassed 5 mM H_2SO_4 at 65 °C. Glucose was quantified using the integration of the peak at 8.9 min and compared to standard curves within in the range 0.5 – 50 g/L. Sugar uptake was assumed to be first order and rate constants were calculated according to literature methods [21].

2.4 Individual inhibitor cultures

The growth of the three yeasts when single inhibitor compounds were present (furfural, 5-HMF, acetic acid, formic acid, levulinic acid) were examined under aseptic conditions using a 96-well microtitre plate system. 200 µl of culture were made up in 96-well plates in YM medium containing 30 g / L glucose and either a low, medium or high level of inhibitor (given in Table 3) sterilised by UV for 1 hour prior to inoculation. The cultures were incubated at 20 °C, 180rpm the increase in the $O.D._{600nm}$ was measured after 168 hours using a plate reader (Versamax, Molecular devices UK).

2.5 Alternative sugar cultures

The performance of the yeasts was assessed on different sugar sources (glucose, glycerol, xylose, arabinose, cellobiose, lactose, sucrose and combinations thereof) using a 96-well microtitre plate system. Briefly, 200 µl of culture were made up in 96-well plates in RM medium containing 30 g / L of the sugar. After 120 days at 20 °C, 180rpm, the $O.D._{600nm}$ was measured using a plate reader (Versamax, Molecular devices UK). Each combination examined was run with six repeats.

2.6 Depolymerised *Miscanthus* and Wheat straw

The *Miscanthus gigantus* grass and wheat straw used for this experiment was depolymerized using two methods. For method one, 5% mass fraction of *Miscanthus* or wheat straw was autoclaved for 2 hours in 0.8% sodium sulphate solution (Na_2SO_4), and the pH was then adjusted to 5.5. The soluble fraction was then hydrolysed with the enzyme CTEC-2 for 72 h. In the second method the *Miscanthus* was soaked in ammonium hydroxide for 24 h prior to the depolymerisation. After 24 h the *Miscanthus* was autoclaved (5% mass) for 2 h in 0.8% sodium sulphate solution (Na_2SO_4), and the pH adjusted to 5.5. The soluble fraction was then hydrolysed with the enzyme CTEC-2 for 72 h. The solutions were all autoclaved at 120 °C for 15 min to ensure sterile conditions prior to inoculation.

2.8 Depolymerisation of municipal food waste

A standard food waste was developed with consumables purchased from a local supermarket. This consisted of: 263 g of boiled carrot, broccoli and cauliflower mix, 357 g of boiled baking potato (skin on), 95 g apple, 65 g orange peel, 150 g banana, 100 g sliced white bread, 100 g sliced cooked chicken, 50 g packet salad, 160 g used tea bags and 90 g of a ready-prepared chilled lasagne. These were macerated using a conventional food processor, until a homogenised substrate was obtained. This mix was blended 1:1 (w/w) with deionised water using a household blender.

Acid hydrolysis was performed according to a modified method of Chi *et al.* [22], in which 3% (v/v) concentrated sulfuric acid was added to the blended food mixture and autoclaved (121°C, 15 min) to hydrolyse the polysaccharides within the food waste. The autoclaved mixture was filtered twice through a Whatman No. 1 filter paper (Whatman, Maidstone, UK) before being neutralised with sodium hydroxide to pH 6.5. The resulting precipitate was again filtered twice through Whatman filter paper to yield a translucent food waste hydrolysate (FWH), which was autoclaved (121°C, 15 min) to ensure sterility. The sugar in the food waste was analysed by HPLC. The water prepared food waste was prepared in the same manner without the addition of sulfuric acid.

2.8 Culture on depolymerised lignocellulose

Rhodotorula minuta (10%) was used to inoculate 20ml of each of these *Miscanthus* and wheat straw hydrolysates. The cultures were held for 168 h, at 28°C and 180rpm in duplicate. 0.5 ml samples were taken every 24 h to measure the OD_{600nm} and assess the sugar and nitrogen concentrations.

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For the one stage process, the food waste hydrolysate (FWH, 20 ml) was inoculated with *Rhodotorula minuta* (10%). Additionally glucose (30 g / L) was added to the food waste (FWH+glucose), or additional salts (yeast extract, 1 g, NaCl 0.1g, KH_2PO_4, 0.4g, $MgSO_4 \cdot 7\ H_2O$, 0.5g, $CaCl_2$, 0.1g, $(NH_4)_2SO_4$, 0.5g, glucose 30 g/L) were added to the foodwaste (FWH + salts). The cultures were run in triplicate, held for 120 h at 28 °C and agitated at 180rpm, the OD_{600nm} was measured throughout the cultures. For the two step process, the cell mass was centrifuged (2000 rpm) after 4 days and the cells agitated at 180 rpm in a glucose only solution (30 g/L) for 48 h at 28 °C.

2.9 Lipid extraction

Lipid was extracted by an adapted literature method first reported by Bligh and Dyer [19]. The samples were stirred in a $CHCl_3$ and MeOH mixture (2:1 v/v) over 48 h, the cell mass was filtered off and washed with additional $CHCl_3$. This was repeated three times. The volatiles were removed under reduced pressure and the lipid analysed. The lipid content and fatty acid methyl ester (FAME) profile were calculated by GC-MS calibrated to known standards. The GC-MS analysis was carried out using an Agilent 7890A Gas Chromatograph equipped with a capillary column (60m × 0.250mm internal diameter) coated with DB-23 ([50%-cyanpropyl]-methylpolysiloxane) stationary phase (0.25µm film thickness) and a He mobile phase (flow rate: 1.2ml/min) coupled with an Agilent 5975C inert MSD with Triple Axis Detector. A portion of the biodiesel samples (approximately 50mg) was initially dissolved in 10ml dioxane and 1µl of this solution was loaded onto the column, pre-heated to 150°C. This temperature was held for 5 min and then heated to 250°C at a rate of 4°C/min and then held for 2 min.

3 Results

The production of cell mass was seen to be largely independent of the level of nitrogen in the culture medium, though temperatures between 25 °C and 28 °C with a glucose loading over 20 g / L gave the maximum growth of just over 8 g / L after 120 h. This was slightly lower than observed for alternative strains in the published literature [19]. The lipid accumulation in *R. minuta* is heavily dependent on a range of environmental factors (Fig. 1). The highest lipid accumulation, of 37% of the cell weight was observed at a high carbon-to-nitrogen ratio (a glucose loading of 30 g / L with 0.5 g NH_4SO_4).

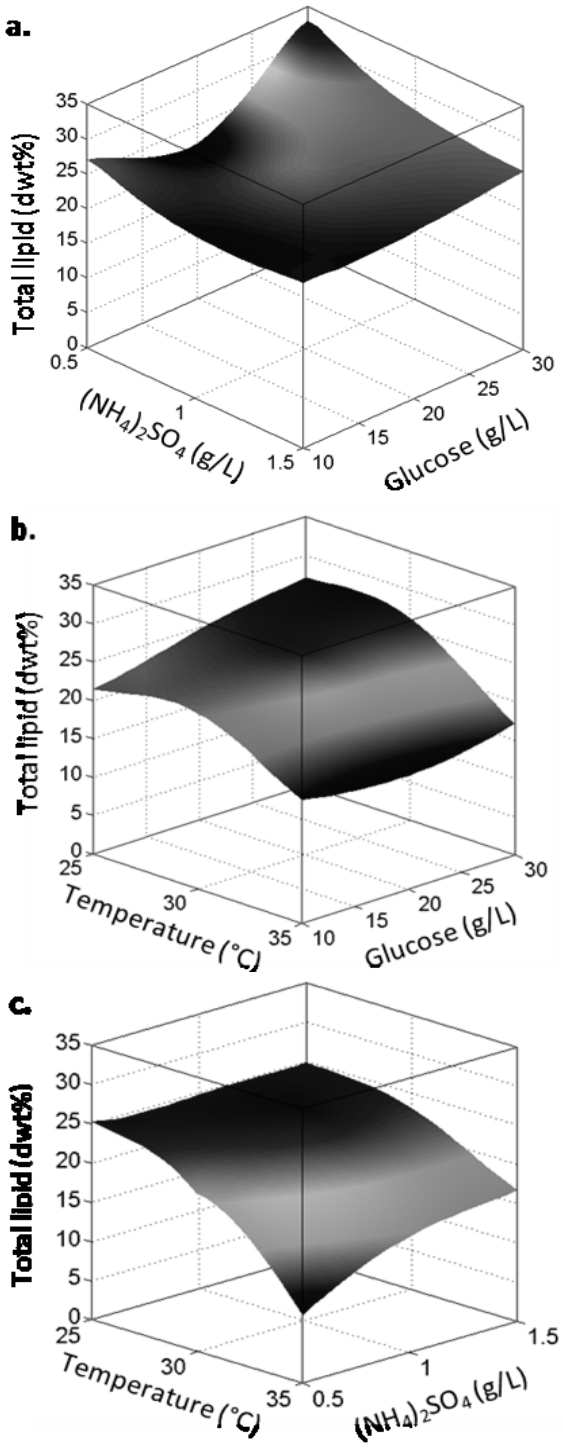

Figure 1 Response surfaces for the total lipid accumulation in *R. minuta,* where a) shows the interaction between glucose and $(NH_4)_2SO_4$, 25 °C b) the interaction between temperature and glucose and c) the interaction between temperature and $(NH_4)_2SO_4$.

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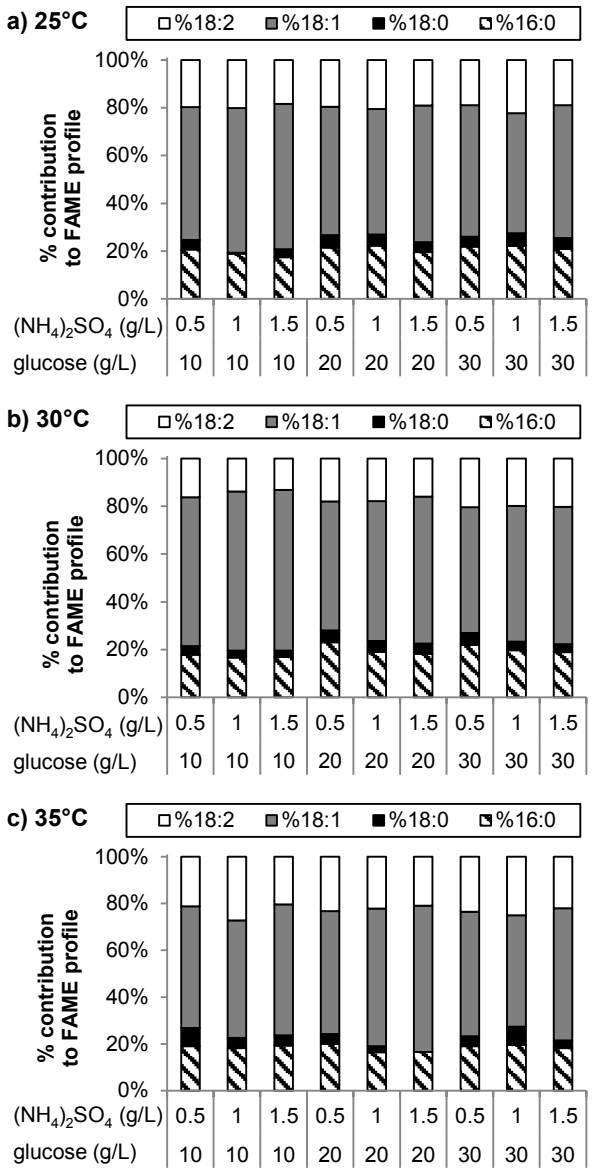

Figure 2 Effect of the glucose and nitrogen loading on the fatty acid profile obtained from the lipid at a) 25 °C, b) 30 °C and c) 35 °C

The relationship between the lipid production and temperature, however, is more complex. At high temperatures low lipid levels were observed irrespective of the other conditions, though there was little difference in a temperature of 25 or 30 °C. Somewhat surprisingly the fatty acid profile did not change significantly on changing the environmental conditions (Fig. 2). The fatty acid profile consisted of 4 major fatty acids, with roughly 16% linoleic acid, 62% oleic acid, 4% stearic acid and 18% palmitic acid. The fatty acid composition is highly similar to rapeseed oil, and would fall within the European standard for biodiesel production [24]. While the cell mass and lipid production can be optimised to produce the highest level, a more suitable metric is the production of cell mass and lipid as a function of sugar consumption (Table 1). The cell mass yield when optimised was near 40% of the sugar. The lipid coefficient, fell between 3.2% and 11.6%. The optimal conditions of cell mass and lipid productivity were with a relatively low C/N ratio of 20 at 30 °C, this is similar to previous studies that showed the optimal lipid production was achieved at a C/N ratio of 30 [19].

Temp (°C)	Glu (gL^{-1})	$(NH_4)_2SO_4$ ($g\ L^{-1}$)	Cell mass coefficient	Lipid coefficient
25	10	0.5	40.4%	6.2%
25	10	1	40.1%	7.7%
25	10	1.5	41.7%	8.7%
25	20	0.5	39.6%	9.8%
25	20	1	37.2%	10.0%
25	30	0.5	38.4%	8.1%
25	30	1	37.9%	11.4%
25	30	1.5	40.0%	11.4%
30	10	0.5	37.4%	11.6%
30	10	1	36.9%	9.4%
30	10	1.5	38.1%	9.3%
30	20	0.5	36.5%	7.0%
30	20	1	38.2%	11.4%
30	20	1.5	31.0%	6.5%
30	30	0.5	28.7%	10.2%
30	30	1	35.2%	10.1%
30	30	1.5	28.8%	6.5%
35	10	0.5	33.9%	5.3%
35	10	1	30.5%	5.0%
35	10	1.5	31.4%	4.1%
35	20	0.5	34.3%	3.2%
35	20	1	32.7%	5.6%
35	20	1.5	32.9%	4.8%
35	30	0.5	33.7%	11.0%
35	30	1	34.8%	5.8%
35	30	1.5	29.8%	5.7%

Table 1 Yield of cell mass and lipid as a percentage of sugar consumption for *R. minuta* cultured on glucose over 120 hours.

One of the key features for the conversion of depolymerised lignocellulose is the ability to

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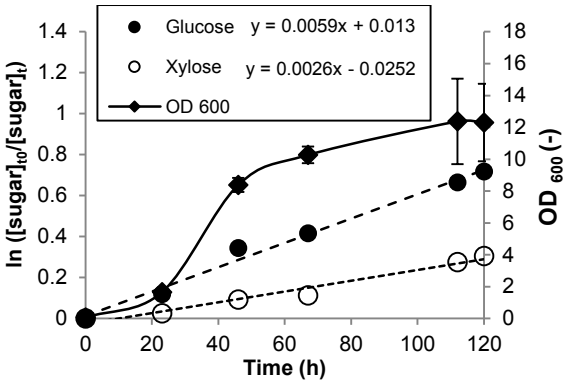

Figure 3 Cell mass productivity and sugar consumption kinetics of *R. minuta* cultured on a 50:50 (wt%) mixture of glucose and xylose at 28 °C, 180 rpm for 120 hours

metabolise C_5 as well as C_6 sugars. While a range of organisms are capable of this, a large proportion will grow diauxicly, utilising the glucose first, then only switch to xylose after an additional lag time. To examine this effect, *R. minuta* was cultured with varying levels of glucose and xylose over 120 hours (Fig. 3, Table 2). Assuming a first order relationship, the rate constant for glucose consumption was found to be 6.5 x 10^{-3} h^{-1} when no xylose was present. The rate of glucose uptake remained reasonably stable until only 25% of the sugar was glucose, it then increased dramatically. The rate of xylose consumption was roughly half that of glucose but remained stable irrespective of the level of xylose in the culture. Xylose was still consumed when glucose is present, starting less than 15 h into the culture. While the growth on xylose is promising a range of other sugars are found in depolymerised lignocellulose.

To assess the suitability of *R. minuta* to be cultured on depolymerised lignocellulose, the yeast was cultured on a range of simple sugars, at 28 °C over 120 h. The growth was compared under identical conditions to that of *R. glutinis* and *Y. lipolytica* (Fig. 4). *R. minuta* grew as well on xylose, arabinose and fructose as on glucose. While *R. minuta* could grow extremely well on the disaccharide cellobiose, it was less effective on lactose and sucrose, resulting in less than 50% of the cell mass observed when grown on glucose. Promisingly, in mixed sugar cultures *R. minuta* grew extremely well on mixtures containing arabinose. The results compare favourably to both *R. glutinis*, that cannot grow on xylose particularly

Table 2 Growth kinetics of *R. minuta* on combinations of glucose and xylose.

Glu (wt.%)	Xyl (wt.%)	*Rate constant of glucose metabolism (h^{-1})*	*Rate constant of xylose metabolism (h^{-1})*
100	0	6.5 x 10^{-3}	-
75	25	5.0 x 10^{-3}	3.4 x 10^{-3}
50	50	5.9 x 10^{-3}	2.6 x 10^{-3}
25	75	9.8 x 10^{-3}	2.7 x 10^{-3}
0	100	1.3 x 10^{-2} [a]	3.0 x 10^{-3}

[a] residual glucose present from the inoculum

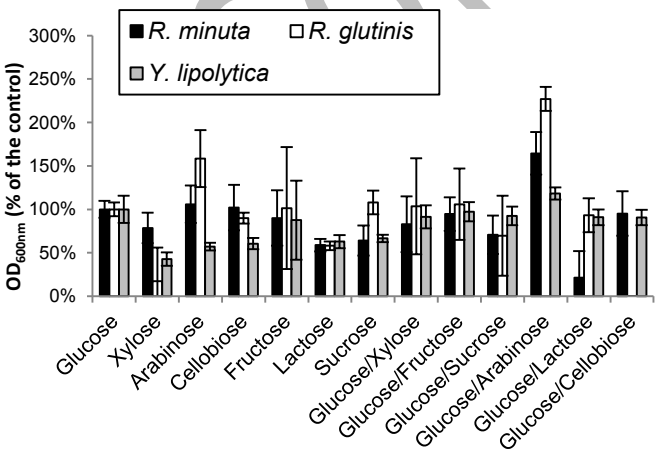

Figure 4 Cell mass productivity for *R. minuta, R. glutinis* and *Y. lipolytica* on a range of simple sugars, O.D.$_{600nm}$ was taken at 120 h. The OD_{600nm} was standardised to a percentage of the growth on glucose. The yeast were cultured on 96 well plates at 28°C. The error bars show the standard deviation from 6 samples.

Table 3 Concentration of inhibitors used in the culturing of *R. minuta, R. glutinis* and *Y. lipolytica* on glucose (30 g/L) in 96 well plates.

Inhibitor	Low value / mmolar (g / L)	Medium value / mmolar (g /L)	High value / mmolar (g / L)
Furfural	1 (	10	60
5-HMF	1	10	60
Acetic acid	10	60	200
Formic acid	10	60	200
Levulinic acid	10	60	200

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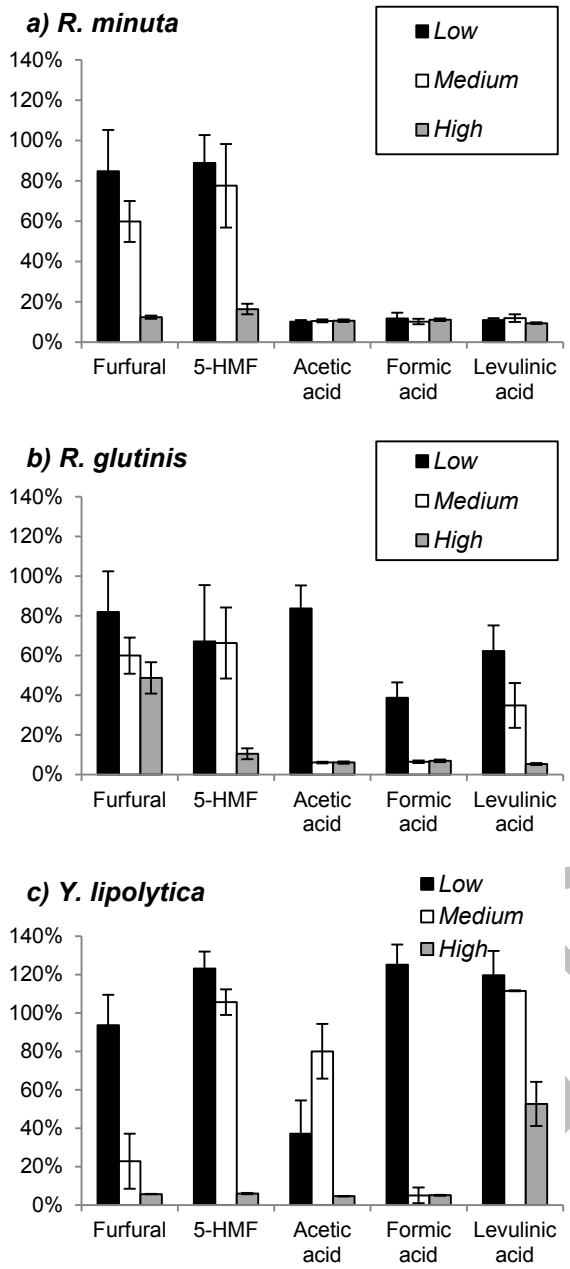

Figure 5 Cell mass productivity in the presence of common inhibitors present in depolymerised cellulose. The OD_{600nm} has been standardised to a percentage of the growth on glucose with no additional inhibitors. The yeasts were cultured at 28 °C, in 96 well plates over 5 days. Error bars represent the standard deviation of 6 identical cultures.

well and with *Y. lipolytica* that grew poorly on xylose, arabinose and the disaccharide sugars. During lignocellulose hydrolysis, the sugars within the cell mass can also degrade into numerous inhibitory products. Glucose can degrade into 5-HMF, levulinic acid and formic acid, whereas xylose will break down into furfural and acetic acid [25].

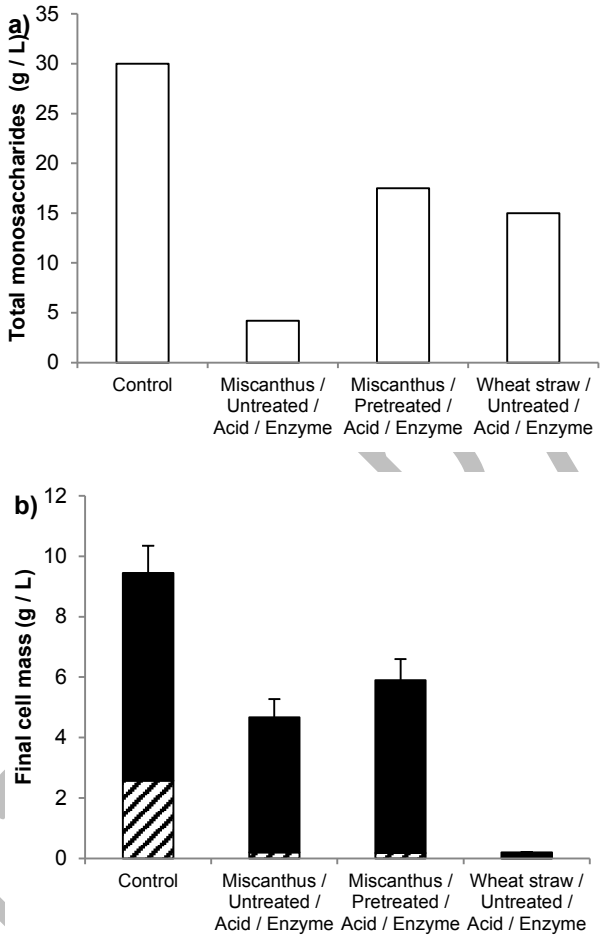

Figure 6 a) Total monosaccharide content in biomass hydrolysate (b) Cell mass and lipid content (shaded) for *R. minuta* grown on depolymerised miscanthus and wheat straw

These inhibitors can have a detrimental effect on the growth and lipid production of oleaginous yeasts. For example, *R. toruloides,* was found to be highly susceptible to furfural concentrations as low as 1mM [26]. To assess the effect of these inhibitors on *R. minuta*, the yeast was cultured on a media with 30g/L glucose and either a low, medium or high level of the various inhibitors (Table 3). The results were normalised to the control (30 g/L) glucose with no additional inhibitors and compared to both *R. glutinis* and *Y. lipolytica* under the same conditions (fig. 5). *R. minuta* can still grow reasonably effectively in the presence of furfural and 5-HMF. Only at high concentrations of these compounds is the growth reduced substantially. However, the acidic inhibitors, acetic acid, formic acid and levulinic acid, affect the growth substantially. In comparison *R. glutinis* demonstrated a similar tolerance to

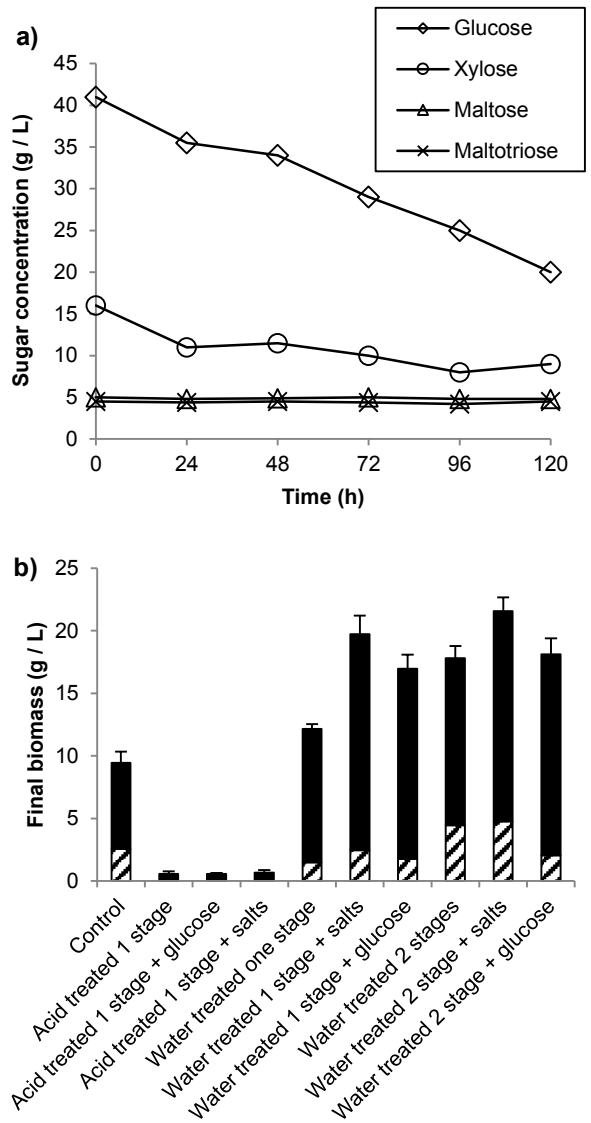

Figure 7 a) Sugar consumption for *R. minuta* cultured on municipal food waste depolymerised through a water treatment step (121 °C, 30 minutes). (b) Final cell mass and lipid (shaded) contents for *R. minuta* cultured on food waste depolymerised through an acid treatment (3% H_2SO_4, 121 °C, 30 minutes), a water treatment (121 °C, 30 minutes) and cultured in a 2 stage process where the yeast was cultured for 4 days on water treated food waste followed by a glucose-only stage for 2 days.

furfural and 5-HMF, yet under low loadings of organic acids still grew reasonably well. A medium or high loading of these acids were highly inhibitory however. *Y. lipolytica* had a similar tolerance to the furfural and 5-HMF as *R. minuta*, yet demonstrated a higher tolerance to organic acids.

Fatty acid	FWH	FWH + glucose	FWH + salts	*R. minuta* control
16:0	25.6	25.61	25.3	21.3
18:0	6.1	6.10	6.7	5.3
18:1	51.2	51.22	58.7	48.0
18:2	17.1	17.07	9.3	25.3

Table 4 Percentage composition for the fatty acid profile for *R. minuta* cultivated in a two stage system with food waste hydrolysate (FWH) and then glucose as the sole carbon source

To examine the suitability of *R. minuta* to be cultured on waste resources, wheat straw and *Miscanthus* were depolymerised according to a multi-stage, chemical pretreatment and enzymatic process (Fig. 6). Firstly the *Miscanthus* was soaked in ammonia overnight before a chemical pretreatment with sulfuric acid (1%). The solubilised feedstock was then depolymerised with cellulases. *R. minuta* growth was severely reduced on the miscanthus hydrolysate, irrespective of whether the biomass had been soaked in ammonia prior to the pretreatment. No growth was observed on culturing using wheat straw hydrolysate. While there was a reduced sugar content in the biomass streams, it was presumably the inhibitors present in the broth that inhibit the growth. An alternative source of suitable biomass is municipal food waste. Food waste is more easily processed than agricultural stover, with a one-step water pre-treatment being sufficient to release enough sugar for culturing while not producing elevated levels of inhibitors.

The depolymerised food waste had high concentrations of suitable sugars, which afforded better growth than the control medium (Fig. 7). *R. minuta* was capable of metabolising both the glucose and the xylose from the hydrolysate though not the more complex oligosaccharides present. The lipid yields varied considerably. For the control culture of *R. minuta,* the dry weight contained 28% lipid which decreased to 10% (d.wt) when the FWH was used (Fig. 7b). Though with a similar level of overall lipid produced from each system. On using a two-step culturing system, with a glucose-only starvation step after the fermentation, the lipid was increased substantially, with 25% (dry cell weight) being produced in the water treated hydrolysate. This reduced lipid production observed with the one-stage system is

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most likely due to a non-optimal C/N ratio in the food waste.

The lipid profiles obtained from all of the cultures were typical of what has been presented previously, consisting of palmitic- (16:0), steric- (18:0), oleic- (18:1) and linoleic acid (18:2). The relative proportions of these lipids did however vary slightly between the yeast cultivated on food waste and the control (table 4). The oil resulting from the food waste hydrolysate had a slightly increased level of 18:1 and 16:0, and a reduced level of polyunsaturated lipids. Most notably, the level of oleic acid (18:1) increased considerably when the yeast was grown on food waste hydrolysate with additional salts. The lipid produced from *R. minuta* cultured on food waste had an 18:1 content similar to that of rapeseed oil (~60%), [27] used for the production of biodiesel in the EU. transesterification of the *Rhodotorula* oil would produce a fuel with similar physical properties, compatible with European (EN 14214) and US (ASTM D6751-08) specifications for biodiesel.

4 Discussion

In this study, *R minuta* was assessed for the ability to produce lipids from lignocellulosic hydrolysate. Initially the production of lipid was assessed using glucose as the carbon source. The total lipid, measured as a function of dry weight, was highest at high glucose and low nitrogen concentrations. This was likely due to the up regulation of the enzyme ATP:citrate lyase which on nitrogen limitation increases the metabolic flux to fatty acid biosynthesis [3]. Similarly to other Rhodotorula species, the lipid production was heavily influenced by temperature, with the lipid content being as low as 5% at 35 °C irrespective of the C/N ratio. The very low cell mass and lipid yields observed at higher temperatures show the importance of controlling temperature in an industrial bioreactor system. Tailoring the fatty acid profile is also important, as this determines the suitable function of the lipids. For example, cocoa butter substitutes and care products require more saturated esters to increase the viscosity [28], while biodiesel fuels require increased levels of monounsaturated and diunsaturated esters to conform with current fuel standards [11]. In alternative yeasts, the fatty acid profile is heavily dependent on the environmental conditions [29]. Similarly yeasts, such as *R. glutinis* and *Y. lipolytica,* demonstrate that the fatty acid profile can be tailored to application by changing the environmental conditions [11,30]. The FAME profile of *R. minuta,* however, is remarkably stable irrespective of the environmental conditions. This is advantageous as a specific formula, and therefore the physical properties of the oil can be guaranteed, this is especially necessary when using multiple streams of cellulosic biomass, where the input variables cannot be controlled sufficiently. The stoichiometry of glucose metabolism indicates a yield of 1.1 moles of acetyl-CoA from 0.56 moles (~100 g) of glucose catabolised [31]. Xylose metabolism can proceed through two mechanisms: the phosphoketolase reaction or the pentose phosphate pathway (PPP). The former is the more efficient of the two, yielding 1.2 moles of acetyl-CoA from 0.66 moles (~100 g) of xylose catabolised, whereas the PPP produces 1.0 mole of acetyl-CoA from the same amount of xylose [31,32]. Assuming that all the acetyl-CoA is directed towards lipid synthesis, a maximum lipid yield of 0.32 g/g and 0.34 g/g can be produced from glucose and xylose, respectively [31]. However, due to other cellular processes requiring glucose, even under ideal conditions for lipid production, the lipid yield on glucose is very rarely more than 0.22 g/g [4,8]. The highest lipid co-efficient obtained was 0.12 g/g for *R. minuta*, this is comparable to the similar *Rhodotorula gramsis* (0.13 g / g glucose), and *Rhodosporidium toruloides* [33, 34]. To achieve elevated lipid co-efficients then tailored strategies, such as fed batch regimes in fully aerated controlled stirred bioreactors are necessary [34-36].

When both glucose and xylose are present in the growth medium, a pattern of diauxic growth can arise in which one substrate is catabolised preferentially over the others, with a lag period occurring between the growth phases [37]. Generally, glucose is utilised during the first growth phase as it can allosterically repress other sugar transporters [35]. Also, the lag phase between growth phases is due to the synthesis of the enzymes necessary for xylose metabolism [37]. This pattern of sequential glucose and xylose uptake was observed in *Lipomyces starkeyi* [38] and *Rhodotorula glutinis* [39]. In contrast, the

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oleaginous yeast, *Trichosporon cutaneum,* was able to assimilate glucose and xylose simultaneously, with no pattern of diauxic growth observed [35]. While the assimilation rate of xylose was lower than glucose, no significant diauxic growth was observed for *R. minuta.* Excitingly, *R. minuta* also demonstrated excellent growth on a range of sugars that are commonly found in depolymerised lignocellulose and alternative waste streams, outgrowing *Y. lipolytica* under the conditions employed.

Acid pretreatment of lignocellulose also releases furans and organic acids, which are generally toxic to a range of microorganisms [40,41]. For example, furfural and 5-HMF have been reported to inhibit alcohol dehydrogenase, pyruvate dehydrogenase and aldehyde dehydrogenase [42]; reducing the supply of NADH and affecting respiration. Furfural can also cause damage to the vacuole, mitochondrial membrane, and actin [43], there is additional evidence that furfural deactivates the ability to replicate cells [25]. *R. minuta* showed an excellent tolerance to both furfural and 5-HMF. *R. minuta* was only inhibited under a 60mM concentration, this is many times higher than would be observed in a bioprocess. Furfural derivatives are generally extremely inhibitory to yeasts [44]. While these compounds were toxic at high loadings, the organic acids were far more inhibitory to the growth of *R. minuta*. Similarly, *R. glutinis, R. rubra, Trichosporon cutaneum, Rhodospridium toruloides* and *Lipomyces starkeyii* have been shown to be severely impacted by ~80mM or less of either formic or acetic acid [45], this also corresponds with the poor growth observed for *R. glutinis* and *Y. lipolytica* at these loadings.

Correspondingly *R. minuta* grew poorly on the *Miscanthus*, wheat straw and food waste hydrolysates treated with sulfuric acid. While to some extent this might be due to the lack of monosaccharides in the hydrolysate most likely the high inhibitor concentrations produced by the pretreatment method restrict the growth. However, municipal waste food is already heavily processed and as such can be depolymerised effectively without additional acids. Accordingly food waste treated with water was found to be an excellent feedstock for *R. minuta*, with far higher growth rates observed than with 30 g/L of glucose. However, reduced lipid levels were produced when the yeast was cultured on this resource alone. Presumably this was due to the low C/N ratio, as when the yeast was starved in a nitrogen free glucose solution for two days the lipid content was increased to 25% dry weight of the cell. The fatty acid profile did not change extensively when cultured on food waste, though when additional salts were present the 18:1 content was higher and the 18:2 lower in comparison to the control. While the environmental conditions do not affect the profile extensively it seems likely that heterogeneous food waste feedstocks potentially could lead to a small variation in the lipid profile.

5 Conclusions

A promising strategy to produce inexpensive microbial lipids is through culturing oleaginous yeast on low-cost resources such as depolymerised lignocellulose or food waste. The growth of microbes on these feedstocks can however be challenging, due to the large range of sugars present in the hydrolysates as well as inhibitory compounds formed during the pre-treatment process. In this investigation we determined that *Rhodotorula minuta* can metabolise a range of sugars present in biomass hydrolysates, and the fatty acid profile of *R. minuta* was found to be stable irrespective of the environmental conditions. This would be highly beneficial when transitioning to waste resources, where the composition of the input stream cannot be guaranteed. However, *R. minuta* was found to be highly susceptible to the inhibitors produced by the acid pre-treatment of lignocellulose. As such, both miscanthus and wheat straw were found to be unsuitable for lipid production with this yeast. In contrast, thermochemically-hydrolysed food waste, with no additional acids, offers great potential for valorisation. On this resource *R. minuta* grew better than on the glucose control, producing up to 25% lipid with the optimal fermentation method.

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Figure legends

Figure 1 Response surfaces for the total lipid accumulation in *R. minuta,* where A) shows the interaction between glucose and $(NH_4)_2SO_4$, B) the interaction between temperature and glucose and C) the interaction between temperature and $(NH_4)_2SO_4$.

Figure 2 Effect of the glucose and nitrogen loading on the fatty acid profile obtained from the lipid at a) 25 °C, b) 30 °C and c) 35 °C

Figure 3 Cell mass productivity and sugar consumption kinetics of *R. minuta* cultured on a 50:50 (wt%) mixture of glucose and xylose at 28 °C, 180 rpm for 120 hours

Figure 4 Cell mass productivity for *R. minuta, R. glutinis* and *Y. lipolytica* on a range of simple sugars, $O.D._{600nm}$ was taken after 5 days. The OD_{600nm} has been standardised to a percentage of the growth on glucose. The yeast were cultured on 96 well plates at 28°C. The error bars show the standard deviation from 6 samples.

Figure 5 Cell mass productivity in the presence of common inhibitors present in depolymerised cellulose. The OD_{600nm} has been standardised to a percentage of the growth on glucose with no additional inhibitors. The yeasts were cultured at 28 °C, in 96 well plates over 5 days. Error bars represent the standard deviation of 6 identical cultures.

Figure 6 a) Total monosaccharide content in biomass hydrolysate (b) Cell mass and lipid content (shaded) for *R. minuta* grown on depolymerised miscanthus and wheat straw

Figure 7 a) Sugar consumption for *R. minuta* cultured on municipal food waste depolymerised through a water treatment step (121 °C, 30 minutes). (b) Final Cell mass and lipid (shaded) contents for *R. minuta* cultured on food waste depolymerised through an acid treatment (3% H_2SO_4, 121 °C, 30 minutes), a water treatment (121 °C, 30 minutes) and cultured in a 2 stage process where the yeast was cultured for 4 days on water treated food waste followed by a glucose-only stage for 2 days.

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